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D-RNAi (Messenger RNA-antisense DNA Interference) as a Novel Defense System Against Cancer and Viral Infections

Shi-Lung Lin[1] and Shao-Yao Ying[2,*]

[1]*Epiclone Inc., 731 South Chapel Avenue, Suite F, Alhambra, CA 91801, USA*

[2]*Department of Cell and Neurobiology, Keck School of Medicine, University of Southern California, BMT-401, 1333 San Pablo Street, Los Angeles, CA 90033, USA*

Abstract: D-RNAi (Messenger RNA-antisense DNA interference), a novel posttranscriptional phenomenon of silencing gene expression by transfection of mRNA-aDNA hybrids, was originally observed in the effects of bcl-2 on phorbol ester-induced apoptosis in human prostate cancer LNCaP cells. This phenomenon was also demonstrated in chicken embryos and a human CD4$^+$ T cell line, H9. The *in vivo* transduction of β-catenin D-RNAi was shown to knock out more than 99% endogenous β-catenin gene expression, while the *in cell* transfection of HIV-1 D-RNAi homolog rejected viral gene replication completely. D-RNAi was found to have long-term gene knockout effects resulting from a posttranscriptional gene silencing mechanism that may involve the homologous recombination between intracellular mRNA and the mRNA components of a D-RNAi construct. These findings provide a potential intracellular defense system against cancer and viral infections.

INTRODUCTION

D-RNAi [Messenger RNA-antisense DNA (mRNA-aDNA) interference] is a process that introduces a hybrid of mRNA and antisense DNA into mammalian cells, resulting in specific silencing of genes homologous to the delivered mRNA-aDNA hybrid [1]. This interference phenomenon of mRNA-aDNA hybrid transfections was recently demonstrated in several biological systems to silence specific gene expression through a type of posttranscriptional gene silencing (PTGS) but the mechanisms by which D-RNAi occurs remain to be elucidated [1]. In addition, ectopic transfection of such an mRNA-aDNA hybrid construct provided a long-term gene knockout effect with high efficacy [1]. Previously, PTGS has been reported in a variety of organisms, including plants [2], *Drosophila melanogaster* [3, 4, 5], *Caenorhabditis elegans* [6, 7, 8, 9], zebrafish [10] and mice [11]. In those observations involving PTGS, the transfection of a plasmid-like DNA structure (transgene) into cells induces typical PTGS phenomena, while that of a double-stranded RNA (ds-RNA) causes an RNA interference (RNAi) effect. The introduction of a transgene or ds-RNA may evoke a process of the intracellular sequence-specific

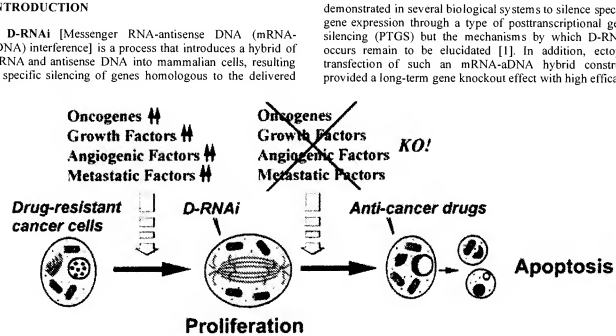

Fig. (1). Schematic illustration of procedures for the application of D-RNAi phenomena in cancer therapy. By reducing drug resistance of cancerous cells, it is possible to apply low dose of chemotherapy to treat and/or cure cancer without side effects.

* Address correspondence to this author at the Department of Cell and Neurobiology, Keck School of Medicine, University of Southern California, BMT-401, 1333 San Pablo Street, Los Angeles, CA 90033, USA; Pone: (323) 442-1859, Fax: (323) 442-3158, E-mail: sying@hsc.usc.edu

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RNA degradation of all highly homologous transcripts that is referred to as cosuppression. In D-RNAi, such cosuppression may be partly responsible for the mechanistic steps of PTGS observed. To the best of our knowledge, D-RNAi is the first observation of a long-term, sequence-specific cosuppression of homologous transcripts in human cells. In this article, we will summarize our recent observations on D-RNAi and outline the strategy for potential development of a novel defense system against cancer (Fig. (**1**)) and viral infections. We also point out the capacity of this technique in determining the functional significance of a specific gene by depleting the gene expression with D-RNAi.

D-RNAi AND ITS MECHANISMS

In a recent report, Grant [2] proposed that the mechanistic effects for PTGS consists of three major steps: initiation, spreading and maintenance. Similar steps of processing were found in several inheritable long-term RNAi phenomena. However, the initiation of D-RNAi involves the onset of a PTGS/RNAi-like response that takes a relatively long time (1-3 days) to generate enough small RNA fragments or short antisense RNAs for specific gene silencing. Although other traditional antisense transfection processes take only a few hours to accomplish the same result, they require a much higher concentration than does D-RNAi. Furthermore, traditional transfections last only 3 to 5 days whereas the effectiveness of D-RNAi remains valid for over 21 days in the transfected cell, which is maintained in the daughter cells but not spread to the neighboring cells. Because there is no spread of the effect to the neighboring cells, the D-RNAi seems to function as an intracellular defense system for eliminating unwanted transgenes and foreign RNAs. In addition to the immune response, this type of new method of defense by D-RNAi in human cells may be useful for the cosuppression of viral infections and retrotransposon activities.

It has been suggested that small RNA products ranging from 21-25 nucleotide bases are generated by an RNA-directed RNA polymerase (RdRp) [2] and/or a ribonuclease (RNase) activity [7, 12, 13], resulting in cosuppression of homologous genes and leading subsequently to the PTGS phenomena. The RdRp-dependent generation of small RNA products resembles that induced by aberrant RNA templates frequently generated from transfection of nucleic acids or viral infection. However, it is not clear whether the small RNAs are directly produced by RdRp [1] or indirectly cleaved from larger RdRp-derived RNA precursors by a specific RNase [7, 12]. On the other hand, an RdRp-independent endoribonucleolysis model was proposed for a short-term RNAi effect in *Drosophilia* [13]. The D-RNAi we discovered [1] is also different from the RdRp-independent endoribonucleolysis model because we transduced only minor concentrations of D-RNAi for a significant long-term effect. For these reasons, the maintenance of such a gene silencing effect must involve an enzymatic amplification process by which the signal of D-RNAi is propagated through several generations of cell replication.

A novel transcription-dependent enzyme was recently determined to be essential for maintaining the D-RNAi effect in humans [1]. This enzyme functions in a manner similar to RdRp homologues found in *Arabidopsi thalianas* as Sde-1/Sgs-2 (14), *Neurospora crassa* as Qde-1 [15] and *Caenorhabditis elegans* as Ego-1 [16]. In our studies with human prostate cancer LNCaP cells, when lower concentrations (1.5-2 μg/ml) of α-amanitin were applied with D-RNAi, the cosuppression phenomena were markedly reduced [1]. We also observed that α-amanitin in concentrations of up to 3.5 μg/ml caused 40-50% transcriptional inhibition without significant induction of apoptosis in the tested cells. Since the α-amanitin is an RNA polymerase II-specific inhibitor derived from a mushroom *Amanita phalloides* toxin, these findings suggest two possibilities. One possibility is that the amplification of D-RNAi signaling requires a relatively high activity of polymerase II-directed transcription machinery in the nucleus. Another is that the Rd Rp enzyme responsible for D-RNAi phenomena is highly susceptible to the α-amanitin toxin. More investigation is being conducted to determine the mechanism of maintenance of a long-term D-RNAi effect.

Alternatively, the homologous recombination between intracellular mRNA and the mRNA components of a D-RNAi construct was found to be involved in the mechanism by which a specific gene is silenced by D-RNAi. We found that the [^{32}P]-labeled aDNA part of a D-RNAi construct was determined to be intact in a hybrid duplex during the effective period of D-RNAi phenomenon, while the labeled mRNA part was replaced by cold homologues and was degraded into small ribonucleotides after a three-day incubation (Fig. (**2A**)). Presently, there is no evidence indicating that these small RNAs can contribute to the gene silencing effect. It is most likely that the D-RNAi construct facilitates the process of nucleotide recombination between the homologous portions of D-RNAi and intracellular mRNAs, and then induces the degradation of all other non-recombinant parts of RNAs as shown in Fig. (**2B**). For these reasons, by eliminating the intact conformation of certain mRNA species, the cosuppression effect of D-RNAi can be useful for silencing specific gene expressions.

THE SILENCING EFFECTS OF ENDOGENOUS GENES BY D-RNAi *IN VITRO*

To examine alternative PTGS/RNAi induction in human cancerous cells, we have tested several different RNA/DNA combination constructs for preventing the expression of specific oncogenic genes. These oligonucleotide constructs include single-stranded antisense DNA (aDNA), double-stranded cDNA (transgene), mRNA-aDNA hybrid (D-RNAi), cDNA-aRNA hybrid (reverse D-RNAi), and double-stranded RNA (RNAi). The sizes of all constructs are larger than 500 bases or base-pairs (bp). Significantly, the results were different from those of RNAi reported from the study with *Drosophila* [13]. At low concentrations (5-25 nM), only the transfection of D-RNAi induced a strong gene silencing effect of interest. For aDNA, a concentration of 500 nM began to show similar effects. Furthermore, concentrations higher than

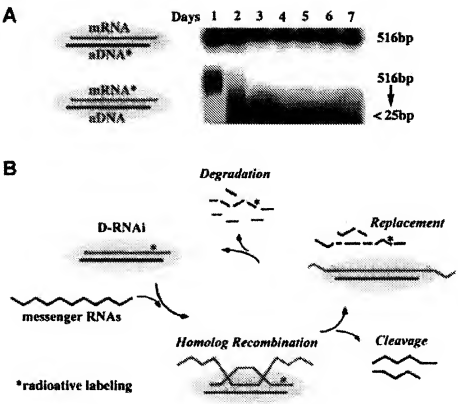

Fig. (2). Potential D-RNAi mechanism for silencing specific mRNA species. (A) The labeled DNA portion was found to be intact during seven-day incubation, while the labeled RNA portion was gradually degraded soon after D-RNAi began to be activated. (B) The oval gray area indicates a homologous sequence between intracellular mRNAs and the mRNA part of a D-RNAi, which facilitates their homologous recombination. After recombination, the mRNA part will be replaced by a new mRNA homologue and then degraded together with non-homologous regions of the intracellular mRNAs.

750 nM for the transfection of each kind of nucleotide construct caused a significant cytotoxic effect and induced cell death. Since the D-RNAi construct cannot be cleaved into the small RNA products that were essential for the endoribonucleolysis observed in RNAi, the mechanical effect of D-RNAi described above may be a novel intracellular mechanism similar to that of the PTGS in plants.

To test this possibility, we took advantage of the fact that most cancerous cells tend to express oncoproteins, which greatly increase their resistance to many chemotherapeutic agents. For instance, over-expression of bcl-2 was known to protect human prostate cancer LNCaP cells from drug-induced apoptosis *in vitro* and to confer resistance to androgen depletion and chemotherapy *in vivo* [17, 18]. The tumorigenic and metastatic potentials were also significantly increased after bcl-2 stimulation. The observed chemotherapeutic resistance can be abrogated by the treatment of conventional antisense oligonucleotides but not those of other apoptotic stimuli such as etoposide or phorbol ester [17, 19]. Unfortunately, conventional antisense treatments require very high dosages (from 1 μM to 40 mM) for a sufficient biological effect that usually results in cytotoxity. For these reasons, we used D-RNAi instead of traditional antisense oligonucleotides for restoring the drug-sensitivity of the cancerous cells to the anti-cancer drugs, e.g., etoposide and phorbol ester (Fig. (1)). Since the effective concentration of D-RNAi is a thousand to million fold less than traditional antisense oligonucleotides, our strategy could provide a much safer and most cost-effective approach for silencing endogenous oncogenes or cancer-growth-related genes.

Our studies in prostate cancer also demonstrated that the transfection of bcl-2 mRNA-cDNA hybrids into LNCaP cells was sufficient to knock out 93% of bcl-2 expression and cause drug-induced apoptosis [1]. Previously, the treatment of androgen withdrawal was shown to stimulate bcl-2 over-expression and inhibit the apoptotic stimuli of phorbol ester. The application of at least 40 mM traditional antisense DNA probes was needed to abolish the inhibition of apoptosis [19, 20]. The effective concentration of D-RNAi for the same bcl-2 knockout effect was more than half-million fold less than those of antisense DNAs. The concentration for 50% cell growth inhibition (IC_{50}) of the D-RNAi was about 750 nM, more than 30 fold of the minimal effective concentration,

indicating that the effectiveness of D-RNAi is not dependent on the amount of its aDNA. Therefore, we postulate that the aDNA part of a D-RNAi construct stabilizes its mRNA half conformation, which significantly enhances the recombinational degradation of targeted gene transcripts. In this way, the effective dosages for antisense gene therapy can be reduced by at least one thousand fold.

A similar strategy can be applied to many other cancer treatments, such as silencing cell-cycle-stimulatory genes to prevent the proliferation of neuronal cancer cells, eliminating local protease activation to reduce cancer metastasis, and knocking out tumor-specific growth factors and/or angiogenic factors to restrict cancer growth. However, because the D-RNAi provides relatively long-term and strong efficacy of homologous gene knockout, it may potentially cause severe damage to the normal tissues. Theoretically speaking, the D-RNAi therapy is not suitable for silencing genes required by both normal and cancer cells. For the same reason, systemic transfection is also not suggested unless a completely transduced animal model is needed for gene function analysis.

THE *IN VIVO* SILENCING EFFECTS OF D-RNAi

Further application of D-RNAi in silencing gene function research was successfully demonstrated in an *in vivo* chicken embryo system. By microinjection of specific mRNA-aDNA hybrids into the amniotic cavity of an E3 (three-day) embryo, a systemic gene silencing effect was detected, resulting in morphological changes in organ development two days after the administration. For example, β-catenin expression is involved in organ formation, particularly in the liver lobe formation during early embryonic development [21]. The transduction of a β-catenin D-RNAi probe interfered with the development of the second and third lobes of the embryonic liver (Fig. (**3**), n = 3). Northern blot analysis of the liver mRNAs demonstrated that β-catenin expression was completely turned off in all three transduced

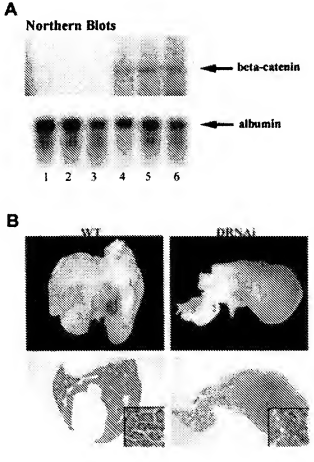

Fig. (3). *In vivo* D-RNAi transfection for specific gene knockout in chicken embryos. (A) Northern blots of extracted RNAs from chicken embryos with (lanes 1, 2 and 3) and without (lanes 4, 5 and 6) β-catenin D-RNAi transfection were shown. All three transfected chicken showed 100% silencing of β-catenin expression but house-keeping genes such as albumin was not silenced. (B) The liver formation of β-catenin D-RNAi transfected chicken embryos was significantly hindered. Microscopic examination revealed a loose structure of hepatocytes, indicating the loss of cell-cell adhesion due to a reported function of β-catenin to E-cadherin in early liver development.

chicken embryos. Because the formation of the first lobe of liver occurs earlier than the time required for the effect of D-RNAi to take place, such developmental regression in the second and third lobes was most likely resulted from a highly stage-specific and long-term effect. The success of this *in vivo* test not only extended the D-RNAi phenomena from mammalian cells to avian embryonic organ development, but also demonstrated the feasibility of D-RNAi in an *in vivo* system.

In addition, these results also shed light on potential functional analysis of novel genes. For these reasons, D-RNAi can be used to screen the functional significance of human novel genes with no known function as identified by the Human Genome Project. For example, recent studies suggested that prostate cancer may be associated with loci in chromosome 1 (1q24-25) and X-chromosome (Xq27-28) [23]; a systemic silencing of the function of genes in 1q24-25 and Xq27-28 as identified by the Human Genome Project can be performed by D-RNAi which may result in the function of genes of interest. Furthermore, because the knockout of an important gene is usually lethal to transgenic mice, the D-RNAi may provide an alternative tool for gene function research in animals other than transgenic mice both locally or systematically.

THE SILENCING EFFECT OF EXOGENOUS GENES BY D-RNAi

Another application of D-RNAi is to silence exogenous genes in a host. Although previous transgene/ds-RNA transfection experiments showed that the PTGS/RNAi effects were limited in plants and some simple animals, we have successfully demonstrated interference of specific gene expression in higher animal models using D-RNAi, including β-catenin gene knockout in chicken embryos and bcl-2 silencing in human prostate cancer LNCaP cells. Since the PTGS/RNAi/D-RNAi is most likely directed to an intracellular defense system against viral infections and/or retrotransposon activities, it is reasonable to design an antiviral drug or vaccine based on the stimulation of this defense system. The cosuppression feature of PTGS/RNAi/D-RNAi helps a transfected cell to develop long-term resistance to viruses with genomic homologues and mutants, which are usually the major problems in the prevention of viral infections. The silencing of both transgene and transposon activities in unicellular alga *Chlamydomonas reinhardtii* has been reported, using a *Mut6* gene, a RNA helicase homologue required for the degradation of certain aberrant/foreign RNAs through PTGS [22]. If this mechanism holds true in mammalian cells, this technique of gene silencing can be used to deplete homologous and mutated viral genes in mammalian cells. As a result, we designed a viral RNA (vRNA)-DNA hybrid sequence to reduce the retrotransposon activity of HIV and observed suppression of the viral transcripts through the D-RNAi mechanism.

Such a cosuppression of HIV homologues may be long-term and inheritably sustained which would be strong enough to eliminate viral genes and/or even their genomes. Although the high mutation rate of HIV genes may induce minor changes of the viral genome enabling it to escape from traditional chemotherapy, it is impossible for HIV to change the targeted sequence that is more than 500 base nucleic acid homologous to a D-RNAi probe. Because of the cosuppression of all homologous transcripts, the HIV genes cannot evade the silencing effect of D-RNAi through mutations. Since the effects of D-RNAi are long-term and perhaps inheritable, this approach could allow development of powerful antiviral drugs or vaccines.

To test the effectiveness of D-RNAi to prevent AIDS, a vRNA-DNA hybrid construct was designed to target a viral gene locus containing 3'-end of *gag*, full-length of *pro* (protease) and 5'-end of *pol* viral genes. As shown in Fig. (**4**) (n = 3), the transfection of this D-RNAi construct into H9, a $CD4^+$ T cell line, completely knocked out the expression of *gag/pol* vRNA precursors at the acute phase of HIV-1 infection, while those of anti-viral DNA, ds-RNA and reverse D-RNAi did not provide a gene silencing effect as

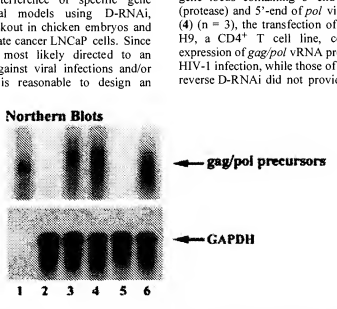

Fig. (4). *In vitro* application of D-RNAi to prevent retroviral infection. Transduction of aRNA-cDNA hybrids (lane 4), mRNA-aDNA hybrids (lane 5) and antisense DNAs (lane 6) was performed in H9 cells, a human $CD4^+$ T cell line, infected with HIV virus for three day. Northern blots of extracted RNAs from the infected cells were performed after one week of incubation. The results showed a 100% knockout effect of targeted HIV-1 genes by the mRNA-aDNA hybrid transfection. No silencing effect was detected in other transfections. Lane 1 is denatured PCR products of *gag/pol* vRNA, while Lanes 2 and 3 are extracted total RNAs from H9 without and with HIV-1 infection, respectively.

determined by Northern blot analysis. Because the foreign viral genes are significantly different from human endogenous genes, these observations indicate that the gene silencing effects by D-RNAi may work on genes of any species with a certain level of homology to the mRNA-aDNA hybrid delivered, but not necessarily to genes in the human genome. Although it is not clear whether the integration of retroviral genome is essential for triggering the D-RNAi propagation, we have observed that the amplification signal from the cell cytoplasm rather than the nucleus is required for a long-term transfection. In addition, we have carried out studies on chronically infected H9 cells and observed a similar silencing phenomenon (Lin *et al.*, unpublished data). These findings further demonstrate a solid potential of using D-RNAi on the design of antiviral drugs. Further study is in progress for an in-depth understanding of the mechanism by which this exogenous gene silencing effect takes place.

DISCUSSION AND CONCLUSIONS

The present article has described a novel method of silencing gene expression in the presence of an mRNA-aDNA hybrid homologous to the silenced gene. Several aspects have been considered in some detail, namely, the D-RNAi phenomenon and the mechanism by which this phenomenon may be involved, the experimental demonstration of bcl-2 oncogene knockout in human prostate cancer LNCaP cells, the developmental retardation of liver lobe formation by silencing catenin genes in chicken embryos, and the interference of viral gene expressions in HIV-infected $CD4^+$ T cells. The D-RNAi acts through a relatively large homolog segment of mRNA (>500 bp), has a long-term effect, functions in mammalian cells and chicken embryos as well as retroviral infections, and passes the effect from original cells to daughter cells. Since this phenomenon can be applied to the interference of genetic abnormality, analysis of gene functions, and removal of exogenous pathogenecity, it has allowed us to develop potential defense against cancers and viral infections. The events evoked by the D-RNAi are relatively easy to detect, whereas the mechanisms involved in its regulation are largely unresolved and remain to be elucidated, particularly the enzymes that contribute to the onset of D-RNAi.

Although we have demonstrated many gene-specific D-RNAi effects under a variety of conditions, including the silencing of bcl-2 and CDC6 in cancerous cell lines, the interference of HIV-1 replication *in vitro* and *ex vivo*, and the knockout of sonic hedgehog, β-catenin and noggin in chicken embryos, none of these experiments showed recoverable results after the overexpression of retroviral carriers of the gene(s). The treatment of recombinant proteins can, however, inhibit the gene silencing effect temporally. It has been noted that the D-RNAi transduction of the reading frame, 3'- or 5'-end of a gene evoked the same effect, thus, there is no significantly fragment-specific changes between these two transductions. Moreover, the transfection of a familial gene usually knocked out the entire homologous members of the gene family, resulting in the silencing of multiple genes. For viral prevention, these features offer panoramic advantages for silencing mutant species, whereas for cancer therapy, greater concerns should be addressed to protect normal tissues. Due to the non-spreading effect of D-RNAi, localization of the treatment within a specific area to reduce the potential risk of affecting surrounding tissues is desirable. Therefore, a more tissue-specific or area-efficient transfection method or vector is required for the clinical use of D-RNAi-like cancer drugs.

The application of D-RNAi technology for pharmaceutical purposes needs more animal tests in various species and human trials to validate its versatile efficacy. Based on current observations, we can only predict its potential applications in the fields of prostate cancer research and retroviral prevention. A highly practical tool for gene function analysis has now been designed for silencing specific gene(s) in chicken embryos and mouse embryonic stem cells by our collaborators. The understanding of distinct gene function in a variety of specific tissues is a tremendous task for completing the human genome projects. With the knowledge of complete gene function, we can then reveal the pathogenic mechanism of certain disease, search for suitable targets for D-RNAi gene therapy, and even manipulate special gene expressions to control organogenesis. Some of these potentials are becoming real while some are still tantalizing. The D-RNAi may provide us a convenient way to explore the unlimited possibilities of gene research faster and clearer.

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